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TITLE: FATTY ACID ETHYL ESTERS OF RHIZOPUS ARRHIZUS

SUBTITLE: Ethyl esters of long-chain fatty acids are reported  
for Rhizopus arrhizus

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ABSTRACT - Gas chromatographic and mass spectrometric analyses on selected lipid fractions revealed for the first time the presence of ethyl esters of long-chain fatty acids as biological products. Ethyl esters of oleic (17.0%), palmitic (1.3%), and stearic (1.3%) acids were detected. Both saturated and unsaturated ethyl esters contain pronounced mass spectral fragments at  $m/e = 88$ .

The presence of naturally occurring esters of short and long-chain fatty acids has been documented for several plant and animal species. The complex epicuticular waxy coating of higher plant surfaces (1,2), insects (3,4), oils of certain aquatic animals (5), as well as the mandibular canal of the porpoise (6) are reported to contain waxy esters with acid and alcohol moieties are reported in a few instances (6). Recently, methyl esters of long-chain fatty acids have been reported for corn pollen (7), chlamydo spores of Ustilago maydis (8), and the sporangiospores and mycelia of Rhizopus arrhizus (9). This article reports for the first time the presence of novel esters of certain long-chain fatty acids containing ethyl groups.

Mycelial fragments (suspended in 0.5 ml of sterile distilled water) were inoculated onto Fothergill's and Yeoman's solid media (10) and grown in the dark at 25° C. After a 3 day incubation period the cultures were harvested. The mycelial mats were immediately frozen, lypholyzed, and 0.434 g were extracted with chloroform: methanol (3:1) followed by n-hexane as previously described (9). The freely extractable lipids were fractionated by silica gel column chromatography and the fatty acid ester components were obtained in the neutral lipid

fraction by elution with benzene. The individual methyl and ethyl esters were separated by gas-liquid chromatography and structure confirmations were made by comparison with authentic standards using mass spectrometry.

As previously reported (9), methyl esters ranging in carbon chain length from  $C_{14}$  to  $C_{24}$  were found in the neutral lipid fraction of total lipid extracts from R. arrhizus. The distribution of methyl esters detected under the conditions presented here is given in Figure 1. No branched chain isomers were detected. After a three day growth period the significant methyl ester components were palmitate and oleate. It was noted that fluctuations in relative concentrations of these esters did occur with time but methyl palmitate and oleate remained the predominant saturated and unsaturated methyl esters. As can be seen in Figure 1, the esters of long-chain acids containing ethyl groups were also significant components. The predominant ethyl esters correspond to the distribution of major free fatty acids typical for this organism (9). Ethyl oleate was the predominant ethyl component (17.0%) in the three day old cultures, whereas ethyl palmitate (1.3%) and ethyl stearate (1.3%) were in lower concentrations. It was observed that relative concentration values changed significantly with age of the culture.

Standards for mass spectrometric comparison were prepared by treating the corresponding fatty acids with  $BF_3$ -ethanol or  $BF_3$ -methanol followed by extraction and gas chromatographic resolution as described previously for the preparation of esters (9). Scans of the natural ethyl esters and the authentic standards produced equivalent spectra.

For ethyl palmitate (Fig. 2) the rearrangement peak  $C_4H_8O_2^+$  at  $m/e = 88$  is the base peak which is analogous to the pronounced  $m/e = 74$  of straight-chain methyl esters. The fact that the base peak shifts from  $m/e = 74$  to  $m/e = 88$  in ethyl esters is due to the ethyl side chain being incorporated in the rearrangement ion formed by 2,3-cleavage. A methoxycarbonyl peak ( $m/e = 101$ ) is also present in ethyl palmitate as illustrated in Fig. 2. The M-45 in this spectrum (which corresponds to the loss of M-31 in methyl esters) and the M-43 are both predominant in ethyl palmitate and have been reported as characteristic peaks for ethyl esters (11). Differences in relative intensities, particularly with respect to the molecular ion, were noted between our data and the values reported earlier by Ryhage and Stenhagen (11). However, the appearance of fragments such as  $m/e = 88$ , 157, 213, and at M-45 and M-43 are intense and apparent in both instances. Differences resulting in a lower contribution by the molecular ion may be attributed to different operational conditions and instrumental characteristics. The  $m/e = 88$  and  $m/e = 101$  peaks are predominant in the monounsaturated ethyl esters as shown in Fig. 3, however, as is the case for methyl oleate,  $m/e = 55$  is also the base peak for the ethyl ester. M-46 represents the loss of ethanol from the monounsaturated ethyl ester and corresponds to the pronounced M-32 observed for monounsaturated methyl esters (12). It seems possible that ethyl esters may be more common as natural products than one might expect because  $m/e = 88$  and  $m/e = 101$  peaks represent major fragments in not only alpha-methyl and beta-methyl branched long-chain methyl esters (13) but also as shown here for long-chain ethyl esters.

The biological role(s) of either methyl or ethyl esters of long-chain acids is obscure at this time but their function may be more than as simple storage products. It has been reported that low concentrations of methyl esters, for example, will enhance auxin and gibberellin activity (14). Volatile constituents of oils containing both methyl and ethyl esters of only low molecular weight fatty acids ( $C_2 - C_8$ ) have been reported using gas chromatography retention data for various fruits such as the pineapple (15). The biosynthesis of methyl esters of long-chain acids has been demonstrated by a soluble enzyme system from Mycobacterium phlei with the methylating agent being S-adenosylmethionine (16). On the other hand, the mechanism of formation of ethyl esters is unknown. The most plausible explanation for the biosynthesis of such esters may be that suggested by Kolattukudy (2) for high molecular weight waxy esters which involve the esterification of free, activated, or protein bound fatty acids with fatty alcohols. Work is now underway in our laboratory to determine the physiological and metabolic significance of these newly observed ethyl esters.

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Figure 1. Gas chromatographic separation of methyl and ethyl esters of long-chain fatty acids of R. arrhizus using a 100 ft x 0.02 in stainless steel capillary column coated with 3% apiezon L. Injector temp 250° C, detector temp. 325° C, temp. program from 130° C - 230° C at 6° C per minute with a 2 minute initial hold.

Figure 2. Low resolution fragmentation pattern of ethyl palmitate at 70 eV. Approximately 85% of gas chromatographic effluent was allowed to enter a Hitachi Perkin-Elmer RMV-6 by means of a glass separator operated at 250° C. The ion source was at 270° C and scans were from 0.0 to 400 m/e in 10 sec.

Figure 3. Low resolution fragmentation pattern of ethyl oleate at 70 eV. Conditions as described in Fig. 2.

RECORDER RESPONSE

FATTY ACID  
ESTERS OF  
R. ARRHZUS

STARTED  
AT 130 °C





